

# **DETERMINATION OF AFLATOXINS IN TRADITIONAL MEDICINE PREPARATIONS**

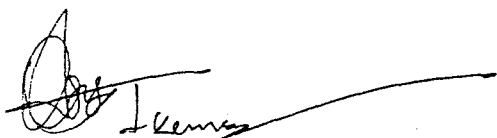
by

OSISI IKENNA ALPHONSE

Thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

April 2003

Saya isytiharkan bahawa kandungan yang dibentangkan di dalam tesis ini adalah hasil kerja saya sendiri dan telah dijalankan di Universiti Sains Malaysia kecuali dimaklumkan sebaliknya. Tesis ini juga tidak pernah diserahkan untuk ijazah yang lain sebelum ini.



Tandatangan Calon

Nama Calon:

Disaksikan Oleh:



Tandatangan Penyelia/Dekan

Cop Jabatan:

PUSAT PENGAJIAN SAINS KIMIA  
UNIVERSITI SAINS MALAYSIA  
11800 USM PULAU PINANG.

## ACKNOWLEDGEMENT

I would like to express my heartfelt gratitude to my parents Mr. and Mrs. Okamigbo Osisi, for their financial support and encouragement throughout the course of my studies. My sincere appreciation also goes to my supervisor Prof. Madya Dr. Bahruddin Saad who made it possible for me to settle down quickly to life in Malaysia, and for his valuable guidance, and direction in the course of my research and for his unwavering kindness. He would always be remembered. My thanks also goes to Dr Norziah Hanni, for providing me with hands-on experience at the beginning of this study, Norhayati Bte Ali, and Dr. Hiroshi Akiyama for providing resource materials at the start of the research. Special mention here must be made of Md Fazlul Bari, for being a friend and counsellor from the first few days of arrival to the present day, and Helal Uddin for his useful contributions and advice.

I would also like to sincerely thank Mr. Ong Chin Hwie, Mr. Yee Chin Leng, Mr Aw Yeong, and Mr. Chow Cheng Por; some of the simplest yet most extraordinary men I have ever met. Their kindness and willingness to help even at short notice would not be forgotten. My study would be incomplete without mentioning the contributions of my class mates, Mr. Chang Seng Teong, Ms. Ng Lee Ping, Ms. Pang Ming Meng and Ms. Tan Sin Ee. Their companionship and sincere friendship went a long way to making me feel at home and I am truly grateful. My gratitude also goes to Ms. Teh Chin Loi and Ms. Lee Ee Lin for their kindness and true friendship.

Finally I would like to thank my siblings for their prayers and support throughout my separation from them. My thanks also to all my friends and acquaintances here in Malaysia, and all member of the FIA research group.

# CONTENTS

	Page
Acknowledgement	ii
Contents	iii
List of Tables	vi
List of Figures	vii
Abstrak	
Abstract	
Chapter 1: General Introduction	
1.1 Mycotoxins	1
1.2 Aflatoxins	3
1.2.1 Sources and Occurrences	3
1.2.2 Chemical Properties	4
1.2.3 Mode of Action	8
1.2.4 Economic and Regulatory Aspects	8
1.2.5 Incidences of Aflatoxin Poisoning	10
1.3 Analytical Methods	13
1.3.1 Immunochemical Methods	13
1.3.2 Thin Layer Chromatography (TLC)	14
1.3.3 Gas Chromatography (GC)	16
1.3.4 High Performance Liquid Chromatography (HPLC)	17

1.4 Detection Methods	17
1.4.1 Ultraviolet (UV) Absorption	18
1.4.2 Fluorescence Detection	18
1.4.3 Electrochemical Detection	18
1.5 Derivatization	19
1.5.1 Pre-column Derivatization	19
1.5.2 Post-column Derivatization	19
1.6 Isolation and Clean-up Methods	20
(i) Liquid-Liquid Extraction	20
(ii) Solid Phase Extraction (SPE)	21
1.7 Objectives of the study	22

## **Chapter 2: Experimental**

2.1 Materials and Methods	24
2.2 Chemicals and Aflatoxin Standards	24
2.2.1 Preparation and storage of aflatoxin standard and sample solutions	25
2.2.2 HPLC	29
2.2.3 Other Apparatus	30
2.2.4 Extraction, Clean-up and Derivatization	30
2.3 Akiyama Method	30
2.4 Modified Akiyama Method	31
2.5 Derivatization Procedure	33

## **Chapter 3: Results and Discussion**

3.1 Modification of the Akiyama Method	34
3.2 Linearity and Detection Limits	35
3.3 Repeatability	36
3.4 Mobile Phase Selection	37

3.5 Recovery studies	41
3.6 Clean-up efficiency for single extract	43
3.7 Clean-up efficiency for multiple extracts	45
3.8 Analysis of Traditional Medicines	47
3.9 Conclusion	53

<b>References</b>	54
-------------------	----

## **Appendices**

Appendix A

Appendix B

## List of Tables

Table 1.1	Conditions for production and the possible involvement of important mycotoxins in human disease	2
Table 1.2	Chemical and physical properties of some aflatoxins and their metabolites	6
Table 2.1	Information on Thai traditional medicines studied	26
Table 2.2	Information on Malaysian traditional medicines studied	27
Table 2.3	Information on Indonesian traditional medicines studied	28
Table 2.4	Information on Nigerian traditional medicines studied	29
Table 3.1	Relative Standard Deviation for Peak Area and Retention Time of Chromatograms for Aflatoxin Spiked Samples Over a 5 Day Period	37
Table 3.2	Percent Recoveries of Aflatoxins Spiked to Traditional Medicine Extracts Using 2 mL of Extracts	42
Table 3.3	Percent Recoveries of Aflatoxins Spiked to Traditional Medicine Extracts Using 1 mL of Extracts	42

## List of Figures

Fig. 1.1	The structure of some aflatoxins and their metabolites	5
Fig. 1.2	Metabolic pathways of AFB <sub>1</sub> in the liver	7
Fig. 2.1	Schematic procedures for analysis of AF modified from the Akiyama Method	32
Fig. 3.1	Typical calibration graph for aflatoxins B <sub>1</sub> , G <sub>1</sub> , B <sub>2</sub> , G <sub>2</sub> using Varian ResElut column (150 x 4.60 mm); CH <sub>3</sub> CN-MeOH-H <sub>2</sub> O (10:20:70)	36
Fig. 3.2	Chromatograms of samples spiked with 10 ppb B <sub>1</sub> /G <sub>1</sub> , and 20 ppb of B <sub>2</sub> and G <sub>2</sub> , using mobile phases of acetonitrile-methanol-water in the ratio (i) 8:27:55; (ii) 17:17:70; (iii) 10:20:70	39
Fig. 3.3	HPLC chromatogram showing separation of aflatoxin standards under optimum chromatographic conditions	40
Fig. 3.4	HPLC chromatogram of underivatised sample extracts (i) before passing through IMC (ii) after passing through the IMC	44
Fig. 3.5	HPLC chromatogram of (i) the first 1 ml eluate (ii) second 1 ml eluate and (iii) third 1 ml eluate passed through the same IMC	46
Fig. 3.6	HPLC chromatograms of (i) underivatised sample extract spiked with aflatoxins, and (ii) derivatised sample extract spiked with aflatoxins	48
Fig. 3.7	HPLC chromatograms of (i) underivatised and (ii) derivatised Thai sample extracts	49
Fig. 3.8	HPLC chromatograms of (i) underivatised and (ii) derivatised Malaysian sample extract	50
Fig. 3.9	HPLC chromatograms of (i) underivatised and (ii) derivatised Indonesian sample extract	51
Fig. 3.10	HPLC chromatograms of (i) underivatised and (ii) derivatised Nigerian sample extract	52



## Abstrak

Mikotoksin telah dikenalpasti sebagai satu masalah besar dalam makanan. Terdapat lima jenis mikotoksin yang penting iaitu aflatoksin (AF), okratoksin (OTA), deoksinivalenol (DON), dan/atau nivalenol (NIV), zearelenon (ZEA) dan fumonisin (FM). Campuran aflatoksin yang wujud secara semulajadi didapati karsinogenik terhadap manusia. OTA dan FA mungkin adalah karsinogenik kepada manusia. Antara aflatoksin yang menunjukkan tahap ketoksikan yang berbeza, aflatoksin B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, dan G<sub>2</sub> adalah paling banyak dikaji dengan aflatoksin B<sub>1</sub> dikenali sebagai paling toksik. Beberapa kajian mengenai pencemaran aflatoksin dalam makanan semulajadi telah dijalankan dan didapati aflatoksin menyebabkan masalah yang ketara di seluruh dunia, terutamanya di kawasan tropika seperti Asia Tenggara dan Afrika. Tujuan kajian ini adalah untuk menentukan tahap aflatoksin B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, dan G<sub>2</sub> dalam ubat tradisional dari tiga negara Asia Tenggara dan satu negara Afrika. Kajian ini merupakan kajian lengkap pertama yang dijalankan ke atas pencemaran semulajadi aflatoksin sediaan ubat tradisional. Tahap aflatoksin B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, dan G<sub>2</sub> dalam sediaan ubat tradisional telah ditentukan dengan menggunakan kaedah kromatografi cecair prestasi tinggi (HPLC) fasa terbalik. Terlebih dahulu, sampel diekstrak dengan asetonitril-air (9:1) dan dibersihkan dalam turus ISOLUTE<sup>TM</sup> MULTIMODE (IMC) multifungsi pengektrakan fasa pepejal. Sampel terbitkan pra-turus dengan menggunakan asid trifluoroasetik (TFA) untuk meningkatkan amatan pendaflour B<sub>1</sub> dan G<sub>1</sub> sebelum pemisahan HPLC menggunakan fasa bergerak asetonitril-metanol-air (10:20:70). Ciri utama pemisahan kromatografi ini ialah kewujudan puncak kuat berpotensi mengganggu berdekatan dengan puncak G<sub>1</sub> yang juga dikesan oleh ahli-ahli kajian dalam sampel-sampel tumbuhan lain. Walaubagaimanapun, kehadiran/ketidakhadiran aflatoksin boleh disahkan dengan

membandingkan kromatogram ekstrak-ekstrak yang tidak terbitkan dan terbitkan. Penggunaan turus IMC adalah menarik memandangkan ia melibatkan satu langkah yang mana bahan asing diperangkan sedangkan aflatoxin melalui turus tanpa ditahan. Sampel yang dianalisis termasuk jamu dan makjun. Alfatoxin tidak dikesan pada 35 sampel yang dianalisis. Rolehan semula untuk B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub> dan G<sub>2</sub> adalah 91.4, 92.9, 102.1 dan 90.8% masing-masing apabila 10 ppb (B<sub>1</sub> dan G<sub>1</sub>) dan 20 ppb (B<sub>2</sub> dan G<sub>2</sub>) pakukan kepada 3 jenis ubat-ubat tradisional yang berlainan.

## Abstract

Mycotoxins have been recognized as a substantial problem in foods. There are five important mycotoxins namely aflatoxins (AF), ochratoxins (OTA), deoxynivalenol (DON) and/or nivalenol (NIV), zearelenone (ZEA), and fumonisins (FM). Naturally occurring aflatoxins have been found to be human carcinogens. OTA and FM are possibly carcinogenic to humans. Of the several known aflatoxins exhibiting different levels of toxicity, aflatoxins B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, and G<sub>2</sub> are the most studied, with aflatoxin B<sub>1</sub> being the most toxic. Studies on aflatoxin contamination of natural foods have shown that aflatoxins constitute a significant problem world wide, especially in tropical regions of the world such as Southeast Asia and Africa. The present study is aimed at determining levels of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> in traditional medicine preparations from 3 Southeast Asian countries and 1 African country, and is the first known comprehensive study conducted on the natural contamination of aflatoxins in traditional medicine preparations. Levels of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> in the traditional medicine preparations were determined using reverse-phase high performance liquid chromatography (HPLC). The samples were first extracted with acetonitrile-water (9:1) and cleaned-up on a multifunctional solid phase extraction ISOLUTE<sup>TM</sup> MULTIMODE column (IMC). The samples were pre-column derivatised using trifluoroacetic acid (TFA) to enhance the fluorescent intensities of B<sub>1</sub> and G<sub>1</sub> before their HPLC separation using a mobile phase of acetonitrile-methanol-water (10:20:70). The main feature of the chromatographic separation of these extracts is the presence of an intense potentially interfering peak near the G<sub>1</sub> peak that were also found by other researchers on other plant samples. However, confirmation of the presence/absence of the aflatoxins could

be made by comparing chromatograms of the underivatised and derivatised extracts. The use of the IMC column is attractive as it is a single step SPE where endogeneous components are trapped, while the aflatoxins were unretained and passed through the column. Samples analysed include the popular after-birth medications "jamu", and "makjun". Aflatoxins were not detected in any of the 35 samples that were analysed. Recoveries for B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, and G<sub>2</sub> of 91.4, 92.9, 102.1 and 90.8 % respectively were obtained when 10 ppb (B<sub>1</sub> and G<sub>1</sub>) and 20 ppb (B<sub>2</sub> and G<sub>2</sub>) were spiked to 3 different kinds of traditional medicines.

# CHAPTER 1

## INTRODUCTION

### 1.1 Mycotoxins

Mycotoxins are derived from the secondary metabolites of some filamentous fungi or molds of the *Aspergillus*, *Penicillium*, and *Fusarium* genera. Under suitable temperature and humidity conditions, they may develop on various foods and feeds, causing serious health risks for humans and animals. Although currently more than 300 mycotoxins are known, attention is focused mainly on those that have been proven to be carcinogenic and/or toxic. These include a metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), the most potent hepatocarcinogenic substance known, which has also been recently proven to be genotoxic; *ochratoxin A*, produced by *Penicillium verrucosum* and *Aspergillus ochraceus*, which is known to be carcinogenic in rodents and nephrotoxic in humans; zearalenone, produced by various species of *Fusarium*, in particular *F. graminearum* and *F. culmorum*, which has an estrogenous action and is significantly toxic to the reproductive system of animals; the tricothecenes, a group of numerous metabolites produced by *Fusarium*, *Stachybotris*, and *Cephalosporium* species, which cause mainly dermatotoxicity, immunotoxicity, and gastro-intestinal disturbances; and the fumonisins, produced mainly by *Fusarium moniliforme*, which may induce as well as hepatotoxicity in rats (Pohland & Wood, 1987). More information on some of these mycotoxins are shown in Table 1.1.

The incidence and extent of mycotoxin contamination are strictly related to geographic and seasonal factors as well as cultivation, harvesting, stocking, and transport

been estimated that 25% of foodstuffs currently produced in the world are contaminated by mycotoxins, with serious repercussions on human health and significant financial loss for the various producing sectors. The real impact of these toxins on human health has only recently been recognized. As a consequence, maximum tolerable limits for all potential risky foodstuffs and consistent official control strategies have only rarely been defined on a national level in several countries. (Brera *et al.*, 1998).

**Table 1.1** Conditions for production and the possible involvement of important mycotoxins in human disease (FAO,1997).

Mycotoxin	Commodities Damaged	Fungal Source(s)	Conditions for Toxin Production	Effect Of Ingestion
Deoxynivalenol and Nivalenol	Wheat, maize, barley, rice, rye, oats, walnuts.	<i>Fusarium graminearum</i> <i>Fusarium crookwellense</i>	High humidity ~20-25°C for growth. ~15°C for toxin production	Human toxicosis. Toxic to animals, especially pigs.
Zearalenone	Maize, wheat.	<i>Fusarium graminearum</i> <i>Fusarium crookwellense</i>	High humidity ~20-25°C for growth. ~15°C for toxin production	Identified by International Agency for Research on Cancer (IARC) as a possible human carcinogen. Affects reproductive system in female pigs.
Ochratoxin A	Barley, wheat, coffee beans, ham, dairy products and many other commodities.	<i>Aspergillus ochraceus</i> <i>Penicillium verrucosum</i>	Relatively non-specific	Suspected by IARC as human carcinogen. Carcinogenic in laboratory animals and pigs. Balkan nephropathy, renal tumors.
Fumonisin B <sub>1</sub>	Maize.	<i>Fusarium moniliforme</i> plus several less common species	Heat and humidity similar to that for AF production	Suspected by IARC as human carcinogen. Toxic to pigs and poultry. Cause of equine leukoencephalomalacia (ELEM), a fatal disease of horses.
Aflatoxins B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> & G <sub>2</sub>	Maize, peanuts, edible nuts, cottonseed, cereals, spices, Fruits, Feeds, Animal products.	<i>Aspergillus flavus</i> <i>Aspergillus parasiticus</i>	Warmth and moisture, High rainfall, High humidity, High temperature. (25-30°C)	Aflatoxin B <sub>1</sub> , and naturally occurring mixtures of aflatoxins, identified as potent human carcinogens, by IARC. Liver cancer, acute hepatitis, reye syndrome. Adverse effect in various animals, especially chickens.

## 1.2 Aflatoxins

### 1.2.1 Sources and Occurrence

Aflatoxins are highly toxic metabolites (mycotoxins) produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. They can be found in a wide range of food items and are potentially hazardous to humans and animals (Stroka *et al.*, 2000). The occurrence and magnitude of aflatoxin contamination varies with geographical and seasonal factors, and also with the conditions under which a crop is grown, harvested, and stored. Crops in tropical and subtropical areas are more subject to contamination than those in temperate regions, since optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. Toxin-producing fungi can infect growing crops as a consequence of insect or other damage, and may produce toxins prior to harvest, or during harvesting and storage.

Aflatoxins have been found in various agricultural commodities, such as peanuts, pistachios, figs; grains, such as corn, rice and wheat; and spices such as paprika (Stroka, *et al.*, 2000). Other food products in which aflatoxins have been found include beans, coffee, eggs, and beer (Jaimez *et al.*, 2000). Four compounds produced by these moulds are aflatoxins B<sub>1</sub> (AFB<sub>1</sub>), B<sub>2</sub> (AFB<sub>2</sub>), G<sub>1</sub> (AFG<sub>1</sub>), G<sub>2</sub> (AFG<sub>2</sub>), (Fig.1.1). Together, these four compounds occur as the major highly active constituents of the *Aspergillus flavus* species. Although 17 aflatoxins have been isolated, the term aflatoxin usually refers to these four compounds. 'B' and 'G' refer to the blue and green fluorescent colours produced by these compounds under ultraviolet light illumination on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Residues of AFB<sub>1</sub> can occur in animal products, including egg, milk and milk products. AFM<sub>1</sub> is also found in human milk as a function of the dietary exposure of the mother to AFB<sub>1</sub>. High

levels of aflatoxin contamination have been found especially in tropical and sub-tropical countries, particularly in Africa and Southeast Asia regions (WHO, 1999).

Aflatoxin B<sub>1</sub> is the most carcinogenic and most commonly occurring variety (Kussak *et al.*, 1995a). It is metabolized in the liver to 2,3-epoxide, which is responsible for alkylation of cellular nucleic acid (DNA) and subsequent carcinogenic and mutagenic activity. Under certain conditions, the LD<sub>50</sub> (lethal dose to cause 50% of mortality after administration in animals) for aflatoxin B<sub>1</sub> was found to be 28 µg in day-old White Peking ducklings (UNEP/WHO, 1979). The LD<sub>50</sub> for aflatoxin B<sub>2</sub> is 85 µg and for aflatoxins G<sub>1</sub> and G<sub>2</sub> they are estimated to be 60-90 µg (UNEP/WHO, 1979).

### 1.2.2 Chemical Properties

The structures of a number of aflatoxins and of aflatoxin B<sub>1</sub> related metabolites are illustrated in Fig. 1.2. The structure of aflatoxins B<sub>1</sub> and G<sub>1</sub> were determined by Asao *et al.*, (1965) and that of B<sub>2</sub> by Chang *et al.* (1963). Aflatoxins B<sub>2</sub> and G<sub>2</sub> are dihydro derivatives of the parent compounds, while aflatoxins M<sub>1</sub> and M<sub>2</sub> are the hydroxylated metabolites of B<sub>1</sub> and B<sub>2</sub>, respectively. Properties of some naturally occurring aflatoxins and metabolites are summarized in Table 1.2.

Aflatoxins are freely soluble in moderately polar solvents (e.g., chloroform and methanol) and especially in dimethylsulfoxide (the solvent usually used as a medium in the administration of aflatoxins to experimental animals). As pure substances, the aflatoxins are very stable at high temperatures, when heated in air. However, they are relatively unstable, when exposed to light, and particularly to UV radiation, and air on a TLC plate and especially when dissolved in highly polar solvents. Chloroform and benzene solutions are stable for years if kept in the dark and cold. Aflatoxins are converted into one or more



stable for years if kept in the dark and cold. Aflatoxins are converted into one or more biologically active metabolites, such as aflatoxicol (AFL) and aflatoxin (AFP<sub>1</sub>) in the liver (Fig.1.1).

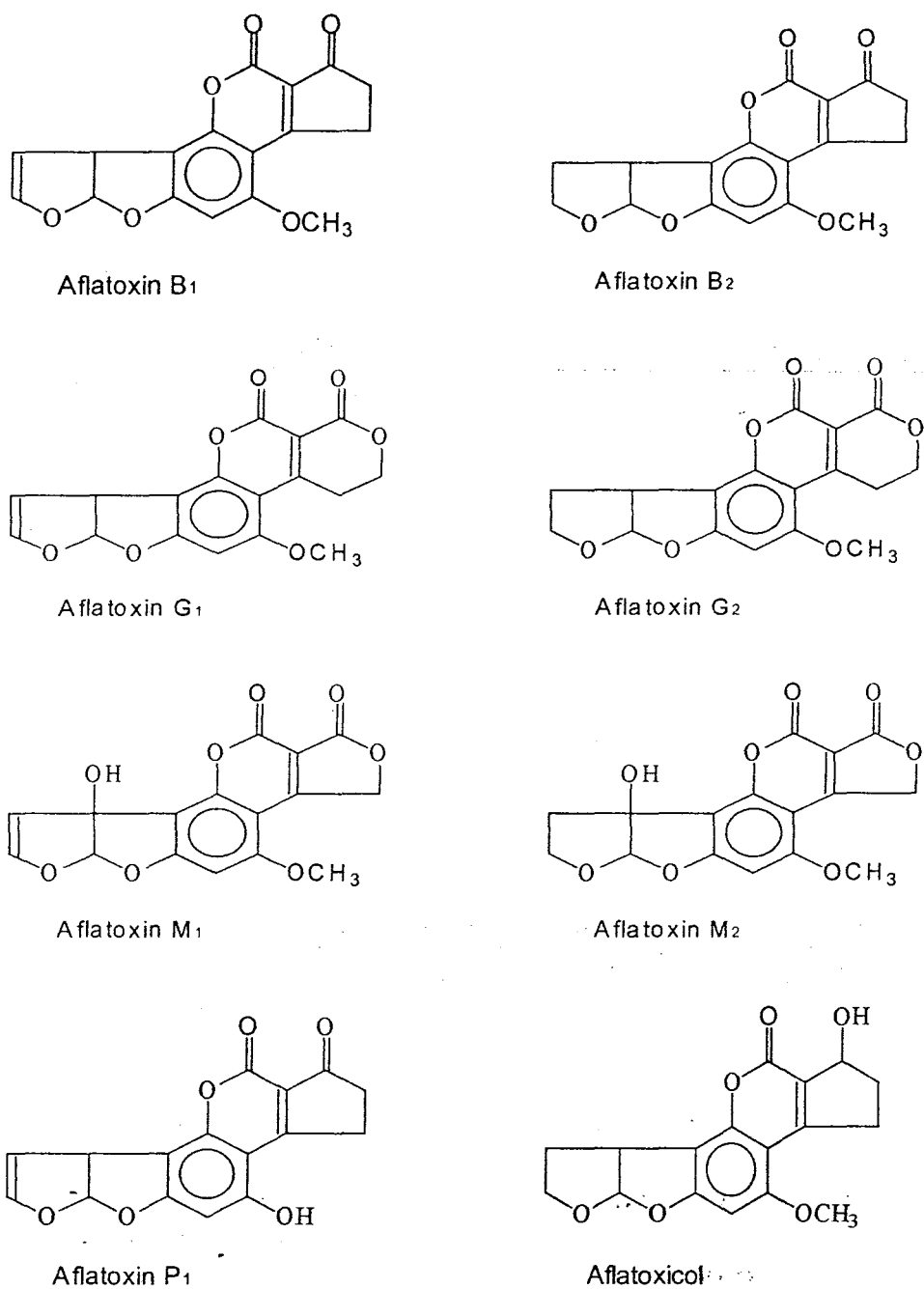


Fig. 1.1 The structure of some aflatoxins and their metabolites

Little or no destruction of aflatoxins occurs under ordinary cooking conditions, and heating for pasteurization. However, roasting groundnuts appreciably reduces the levels of aflatoxins and they can be totally destroyed by drastic treatment such as autoclaving in the presence of ammonia or by treatment with hypochlorite (UNEP/WHO, 1979).

**Table 1.2** Chemical and physical properties of some aflatoxins and their metabolites (UNEP/WHO, 1979)

Aflatoxin	Molecular Formula	Relative Molecular Mass	Melting Point (°C)	Fluorescence Emission	UV Absorption(ε) (360-362nm)
B <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	312	268-269	425	21 800
B <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	314	286-289	425	25 000
G <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	328	244-246	450	17 700
G <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	330	237-240	450	17 100
M <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	328	299	425	21 250 (357) <sup>a</sup>
M <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	330	293	--	22 900 (357) <sup>a</sup>
P <sub>1</sub>	C <sub>17</sub> H <sub>10</sub> O <sub>6</sub>	298	>320	--	15 400 (362) <sup>a</sup>
Q <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	328	-b-	--	17 500 (366) <sup>a</sup>
Aflatoxicol	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	314	230-234	425	14 100 (375) <sup>a</sup>

a. Measured wavelength.

b. Data not available

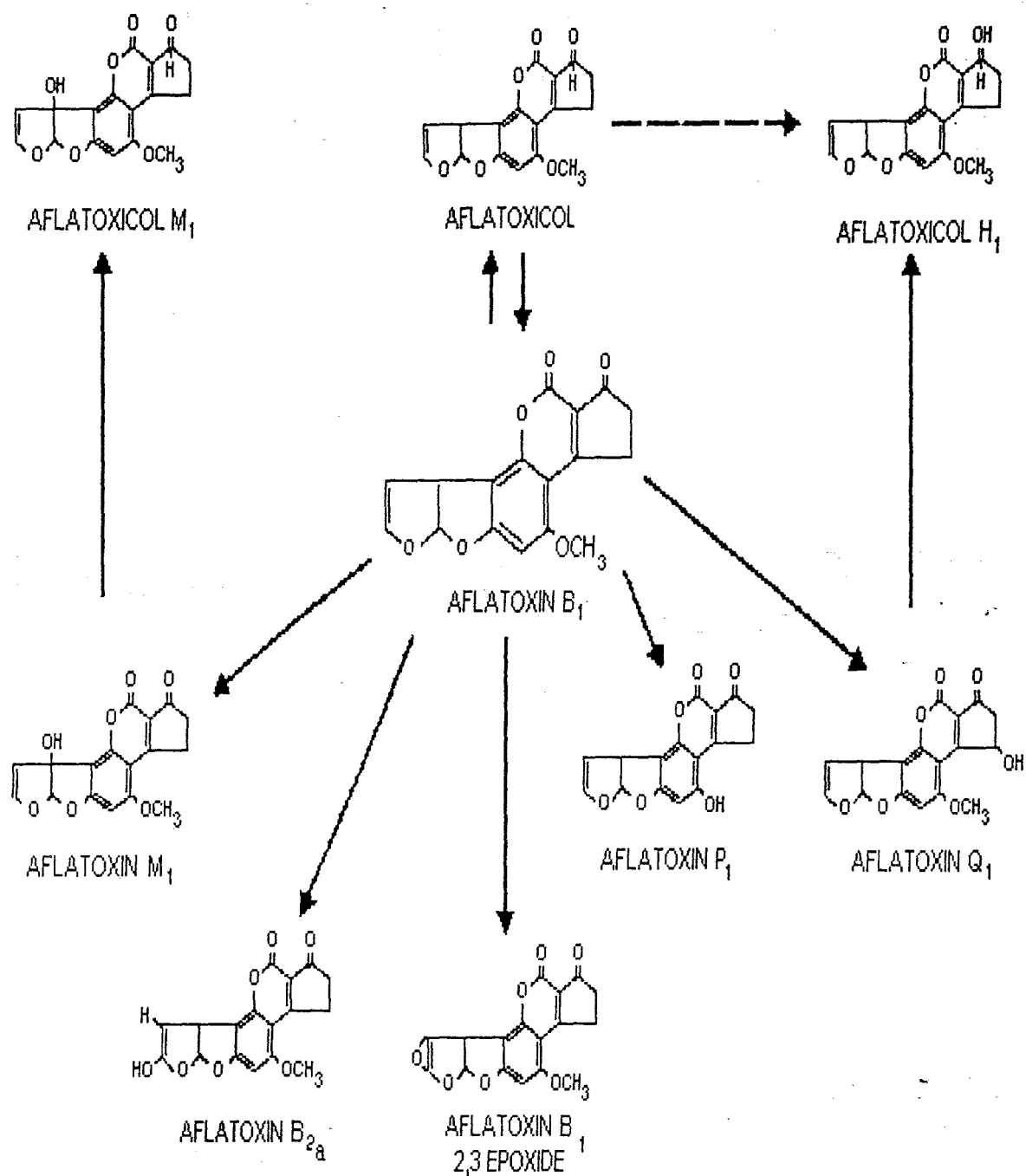


Fig 1.2 Metabolic pathways of AFB<sub>1</sub> in the liver (UNEP/WHO, 1979).

### **1.2.3. Mode of Action**

Aflatoxin B<sub>1</sub> is a liver carcinogen in at least 8 species including nonhuman primates. It acts by causing chromosomal aberrations and DNA breakage in plant and animal cells. After microsomal activation, gene mutations in several bacterial test systems have been reported. In high doses, it may be teratogenic (causing embryo malformation). Dose-response relationships have been established in studies on rats and rainbow trout, with a 10% tumour incidence estimated to occur at feed levels of AFB<sub>1</sub> of 1 µg/kg, and 0.1µg/kg, respectively (UNEP/WHO, 1979). Evidence suggests that aflatoxins are one hundred times more likely to induce cancer than polychlorinated biphenyls (PCBs), and also inhibit the body's immune system and reduce the effectiveness of vaccines (FAO, 1997).

Liver cancer is more common in some regions of Africa and Southeastern Asia than in other parts of the world. When local epidemiological information is considered together with experimental animal data, it appears that increased exposure to aflatoxins may increase the risk of primary liver cancer. (UNEP/WHO, 1979).

### **1.2.4. Economic and Regulatory Aspects**

International trade in agricultural commodities such as wheat, rice, barley, corn, sorghum, soybeans, groundnuts and oilseeds amounts to hundreds of millions of tonnes each year (FAO, 1988). Many of these commodities run a high risk of mycotoxin contamination. Regulations on mycotoxins have been set and are strictly enforced by most importing countries. For some developing countries, where agricultural commodities account for as much as 50 percent of the total national exports, the economic importance of mycotoxin infection is considerable. There is a notable length of time between the purchase of the

agricultural commodity at the village market of the exporting country and its arrival at the distribution centre of the importing country. For these reasons, there is considerable opportunity for mould and mycotoxin contamination of agricultural commodities to take place throughout the food system - from production to distribution and transport - and this may lead to economic losses (Bhat, 1988). The direct cost of aflatoxin contamination on maize and groundnuts has recently been estimated to be greater than \$A470 million per annum, for South East Asian countries, including Australia. Maize was the more important commodity amounting to 66% of the total amount. Indonesia was the worst affected, bearing 48% of the estimated loss. The losses resulting from spoilage accounted for 24% (\$A108 million) per year. (Bhat & Vasanthi, 1999).

Regulations have been set in more than 70 countries in order to restrict the intake of mycotoxins. However, the legal limits vary significantly both from country to country and by mycotoxin type and matrix. For example, limits for aflatoxins in foodstuffs range from 0 to 50 ng/g. The frequent occurrence, particularly of aflatoxins, has already led to temporary bans of certain "high-risk" foods imported into Europe (for example, Egyptian peanuts and Iranian pistachios), where limits have been established at relatively low levels. As a result of the establishment of the EU and its aim of harmonization of the internal market, the European Commission (EC) drafted regulations concerning certain contaminants. The current maximum levels for aflatoxins set by the EC are 2 ng/g for AFB<sub>1</sub> and 4 ng/g for total aflatoxins in groundnuts, nuts, dried fruits and cereals. These are to be extended to cover spices with limits of 5 ng/g and 10 ng/g for aflatoxin B<sub>1</sub> and total aflatoxins, respectively. These levels are about five times lower than those established in the USA (EU, 1998). In Germany, the limits are 4 ppb (w/w) for the sum of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and

G<sub>2</sub> and 2ppb for aflatoxin B<sub>1</sub>. For human dietary products, such as infant nutriment, there are stronger legal limits: 0.05 ppb for aflatoxin B<sub>1</sub> and the sum of all aflatoxins. Indonesia at present proposes a maximum level of 50 ng/g for total aflatoxin in feedstuff. The Philippines and Thailand have an authorized action level of 20 ng/g, while Malaysia has regulated a maximum level of 35 ng/g for total AF in foods (Ali *et al.*, 1998).

#### 1.2.5 Incidences of Aflatoxin Poisoning

The carcinogenicity of aflatoxins have been implied in reports which suggest that some outbreaks of sub-acute poisonings resulting from ingestion of large amounts of aflatoxins over a period of time, with some of the poisonings resulting in fatalities.

##### Malaysia (1988)

An outbreak of food poisoning resulting in 13 deaths in children occurred in Malaysia during the Chinese Festival of the Nine-emperor gods in 1988. The offending food was a Chinese noodle called 'Loh See Fun' (LSF). The source was traced to a factory where a banned food preservative (boric acid) was added to make the LSF. In addition, high levels of aflatoxins up to 3465 pg/g of tissue for B<sub>1</sub>, 9116 pg/g of tissue for G<sub>1</sub>, and 18,521 pg/g of tissue for M<sub>1</sub> were found in the liver of some of the patients when autopsies were conducted, (Chao *et.al*, 1991).

### India (1974)

In the fall of 1974, an epidemic occurred in more than 150 villages in adjacent districts of two neighboring states in a rural area of Northwest India. The disease was characterized by onset with high fever, rapidly progressive jaundice and ascites. According to one report of the outbreak, 397 persons were affected and 108 people died. One notable feature of the epidemic was that it was heralded by the appearance of similar symptoms in the village dogs. Analysis of food samples revealed that the disease outbreak was probably due to the consumption of maize heavily infested with the fungus *Aspergillus flavus*. Unreasonable rains prior to the harvest, chronic drought conditions, poor storage facilities and ignorance of dangers of consuming fungal contaminated food all seem to have contributed to the outbreak. The levels of aflatoxin in food samples consumed during the outbreak ranged between 2.5 and 15.6 µg/g. Anywhere between 2 and 6 mg of aflatoxin seems to have been consumed daily by the affected people for many weeks. In contrast, analysis of corn samples from the same areas the following year (1975) revealed very low levels of aflatoxins (i.e. less than 0.1 µg/g ), and this may have explained the absence of any reoccurrence of the outbreak in 1975 (Krishnamachari, 1975).

### Kenya (1981)

Between March and June 1981, 20 patients (8 women and 12 men aged 2.5 to 45 years old) were admitted to hospital in the Machakos district of Kenya with severe jaundice. The patients reported that they had first exhibited symptoms of abdominal discomfort, anorexia, general malaise and low-grade fever. After about 7 days, jaundice and dark urine appeared,

and the patients sought admission to hospital. Interestingly, the relatives and friends of one family told that many of the local doves had died, then the local dogs, and finally the people had become sick. The dogs were known to be consuming essentially the same diet as the local people. On admission to hospital, all patients were jaundiced, some with low grade fever, and extremely weak, Tachycardia and oedema (of the legs and to a lesser extent the face and trunks) were seen. Eight of the 20 patients improved with a return of appetite, and were discharged from hospital in 20 days. However, hepatic failure developed in the remaining 12 patients and they died between 1 and 12 days following the admission. An extensive investigation of the outbreak was performed. Aflatoxin levels in foods were measured and showed high levels of aflatoxin B<sub>1</sub> and B<sub>2</sub>. For example, maize grains from the two homes where severe and fatal illness had occurred contained 12 mg/kg and 3.2 mg/kg of aflatoxin B<sub>1</sub>, while maize from unaffected homes had a maximum of 0.5-mg/kg aflatoxin B<sub>1</sub>. The cumulative evidence suggests that aflatoxin poisoning was the cause of the acute liver disease in this incident (Ngindu *et.al* 1982).

### Germany

A case has been reported of a 45-year-old man, who died shortly after an apparent gastric illness after eating an unusually large amount of nuts, which apparently were quite mouldy. Analysis of his liver revealed the presence of a blue fluorescing material, which was suspected to be AFB<sub>1</sub>, suggesting acute aflatoxin poisoning (Wilson, 2001).



### 1.3 Analytical Methods

Several analytical methods for the detection of aflatoxins are reported in literature. A discussion of some of the important ones are discussed below.

#### 1.3.1 Immunochemical methods

Aflatoxins can be determined using enzyme-linked immunosorbent assay (ELISA) techniques. Immunoassay has been an important tool for aflatoxin testing since ELISA kits for recognizing different mycotoxins were commercially available. The ELISA format for aflatoxins contains typically three specific reagents: the mono- or poly-clonal antibodies, which recognize and bind with a specific aflatoxin, an aflatoxin-enzyme conjugate and an enzyme substrate. The bonded enzyme catalyzes the oxidation of a substrate to form a coloured complex for further qualitative or quantitative evaluation (Trucksess, *et al.*, 1989; Kussak, 1995b)

The major advantages of the ELISA and affinity column methods include speed, ease of sample preparation, ease of use, and a potentially low cost per analysis. The disadvantages include different antibody specificities for B<sub>1</sub> and cross reactivity with other aflatoxins. ELISA procedures are qualitative or semi-quantitative at best and are temperature sensitive. The major application for ELISA procedures at present is screening for aflatoxin B<sub>1</sub> below a predetermined concentration. The colour developed by the enzyme-mediated reaction gives an indication of the amount of B<sub>1</sub> present. With further development, immunochemical methods will probably become more versatile and suited to a wider variety of applications. (Trucksess, 1989).

### 1.3.2 Thin layer chromatography (TLC)

Many of the older methods adopted by scientific groups and government agencies are based on TLC detection and quantification procedures that have been evaluated in collaborative studies. The AOAC (1984) recommends the contamination branch (CB) and the best foods (BF) methods for aflatoxin analysis in groundnuts.

The CB method (AOAC 1984) is the standard by which other methods are judged. In the CB method, samples are extracted with a mixture of water and chloroform by shaking in a 500 ml Erlenmeyer flask on a wrist-action shaker. The lipids and aflatoxins are transferred to a silica-gel column where the lipids are selectively eluted with hexane and the pigments and other interfering material eluted with absolute diethyl ether. Finally the aflatoxins are eluted from the column with 3 % methanol in chloroform. Though the CB method is an excellent TLC method, it has two major disadvantages: (i) it is expensive because it uses large amounts of solvents, which creates a disposal problem, and (ii) the major solvent used is chloroform, which may be hazardous to workers (Wilson, 2001). Because the CB method is time-consuming, attempts have been made to simplify it. Thus the Best Foods (BF) method was developed, which is faster and more economical in terms of the amounts of solvents used but provides poorer clean-up. The sample is extracted and defatted with a two-phase aqueous methanol-hexane mixture; the aflatoxins are then partitioned from the aqueous phase into chloroform, leaving lipids and pigments in the hexane and aqueous methanol. In both the CB and BF methods, the aflatoxins are concentrated by evaporation of the chloroform, and then separated by thin-layer chromatography (TLC) (AOAC, 1984). To visualize the mycotoxin spots on thin-layer plates, two kinds of techniques have been frequently applied: (i) examination under UV

light of long or short wavelength for naturally fluorescent mycotoxins like aflatoxins, citrinin, ochratoxin A, or (ii) spraying the plates with a chemical reagent (e.g. trifluoroacetic acid, hydrochloric acid, or sodium hydroxide) that reacts with the mycotoxins to produce a coloured or a fluorescent product (Lin *et al.*, 1998). The amount of aflatoxins is then determined by visually matching the intensity of fluorescence of the sample spots with that of standard spots. More accurate quantification of mycotoxins are popularly performed by fluorescence absorbency densitometry with quantification limits ranging from 10 µg/kg down to 0.005 µg/kg levels for various mycotoxins (Kamimura *et al.*, 1985; Trucksess *et al.*, 1984, 1990; Lin *et al.*, 1998)

Kamimura *et al.* (1985) described a simple rapid high performance TLC (HPTLC) method, which compared favorably with the CB method. Trucksess *et al.* (1984) published a rapid TLC method using a disposable silica gel column for clean-up and confirmation by gas chromatography-mass spectrometry (GC/MS). However, no matter which TLC methods are used, the aflatoxin identified needs to be confirmed, using other complementary analytical techniques.

Overpressured-layer chromatography (OPLC) developed by Tyihak *et al.*, (1979) unites the advantages of the HPLC and HPTLC methods. OPLC operates in the same way as regular TLC, but operates in a closed system. Three edges of the plates are closed and the stationary phase is covered with a membrane. A pump is used to supply the mobile phase, which is forced to flow through the stationary phase, located in a closed compartment. Therefore, a constant flow velocity is achieved, optimizing flow velocity and improving efficiency. However, before and after the separation process, OPLC operates in the same way as regular TLC. Aflatoxin analysis based on OPLC separation and

densitometric quantitative evaluation was carried out by Gulyas (1985), and by Otta *et al.* (2000) in the analysis of aflatoxins in agricultural and food samples.

TLC and immunochemical methods may not always be cheaper than HPLC in the long run because HPLC requires a single large initial investment, and TLC and ELISA both use expensive disposable plates. HPLC is possibly more suitable for large analytical laboratories while TLC is more suitable for laboratories with only a few samples to be analysed.

### 1.3.3 Gas Chromatography (GC)

Historically, gas chromatography (GC) was introduced for mycotoxins in the early 1970's. Gas chromatographic (GC) methods have been used, to a limited extent for determination of some mycotoxins like T-2 toxin in grains and animal feeds. In GC, mycotoxins or their derivatives are mostly detected with flame ionization or electron-capture. Goto *et al.* (1988) also reported a method for the determination of aflatoxins in food by capillary column gas chromatography. However, in comparison with TLC, GC method requires that the compounds be of sufficient volatility and relatively non-polar. Mycotoxins, which are not sufficiently volatile, require pre-column derivatization such as silylation or polyfluoroacylation to obtain a more volatile derivative. Apparently, such steps add to the complexity of the procedure (Lin *et al.*, 1998). GC can also be effectively coupled to a mass spectrometer (GC-MS) to obtain qualitative data concerning the identity of the components being analysed (Betina, 1989).

### 1.3.4 High Performance Liquid Chromatography (HPLC)

Aflatoxin analysis using HPLC for separation and detection is quite similar to TLC because similar sampling and extraction procedures are used. However, due to its higher separation power, ease of automation, improved accuracy, and shorter analysis time, HPLC techniques using fluorescence detection has today become the most accepted method for the determination of aflatoxins. Use of HPLC in the analysis of foods for aflatoxins has greatly increased in recent years and several reviews have been made (Jaimez *et al.*, 2000).

Both normal- and reversed-phase HPLC can be utilized in the separation of aflatoxins using both UV and fluorescence detection. Aflatoxins can be resolved by reverse phase HPLC columns with methanol-water or acetonitrile-water, but in these solvents, the fluorescence of AFB<sub>1</sub> and AFG<sub>1</sub> is rather weak, and in order to overcome this problem derivatization is carried out using strong acids such as trifluoroacetic acid (TFA) and oxidants such as chloramines T, iodine, and bromine (Jaimez *et al.*, 2000). Several reverse-phase methods have been published (Cohen & Lapointe 1981) including comparisons to the CB method (Trater *et al.*, 1984). In general, reverse phase HPLC systems are used more frequently than those of normal-phase due to the easier manipulation as well as the lesser toxicity of the mobile phases used.

### 1.4 Detection Methods

Reverse phase HPLC has been used in combination with many types of detectors; the major ones are discussed next.

### **1.4.1 Ultraviolet (UV) Absorption**

Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, which are usually determined in foodstuffs, possess strong UV absorption in the region of 360 nm (in methanol) with extinction coefficients ranging from 17,700 (for G<sub>1</sub>) to 24,000 (for B<sub>2</sub>) (Lin *et al.*, 1998).

### **1.4.2 Fluorescence Detection**

Aflatoxins are intensely fluorescent when they are irradiated under long-wave UV light, thereby facilitating foodstuff analysis. Fluorescence detection, under optimum conditions, is about 30-40 times more sensitive than UV detection for aflatoxins (Elizade-Gonzalez *et al.*, 1998), with quantification limits ranging from 10 mg/ kg down to 0.005 mg/ kg (Lin *et al.*, 1998). Currently, reverse phased HPLC in conjunction with fluorescence detection is the most popular analytical method for aflatoxin analysis.

### **1.4.3 Electrochemical Detection**

HPLC with electrochemical detection has become popular for trace analysis due to its high sensitivity and excellent selectivity. Pulsed amperometric detection is carried out using various types of electrodes such as platinum, gold, and glassy carbon. A reverse-phase-HPLC method with amperometric detection using a glassy carbon electrode has recently been reported for the separation and identification of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub> with

sensitivity of between 7 and 10 ng for the different aflatoxins (Elizade-Gonzalez *et al.*, 1998).

## **1.5 Derivatization**

### **1.5.1 Pre-column Derivatization**

Fluorescent detection of aflatoxins can be enhanced considerably with the formation of derivatives; and the most common method of derivatization is by pre-column derivatization, leading to the formation of derivatives, and consequent enhancement in signals. A comparison of pre- and post-column derivatization methods using HPLC show that the detection limit of almost three times lower ( $0.3 \mu\text{g/kg}$  for aflatoxin B<sub>2</sub>) was reached by pre-column derivatization (Elizade-Gonzalez *et al.*, 1998).

### **1.5.2 Post-column Derivatization**

Different methods of post-column derivatization have been developed. Davis and Diener (1980) reported that AFB<sub>1</sub> and AFG<sub>1</sub> give some intensely fluorescent derivatives with iodine. These authors developed chromatographic analysis of these AFB<sub>1</sub> derivatives with iodine and bromine. Although good results have been reported, halogenated systems present several drawbacks, namely: (i) extended time to stabilize the mobile phase of at least 1 hour, (ii) thermostating the column at 75°C, (iii) dilution caused by reagent addition; (iv) the iodine solution must be prepared daily for suitability reasons, and (v) an additional

pump is needed. Use of very saturated solutions contributes to the great physical and mechanical deterioration of the connection tubing and the post-column pumping device suffers, owing to its prolonged contact with iodine (Jaimez *et al.*, 2000).

## 1.6 Isolation and Clean-Up Methods

Generally three steps are involved in mycotoxin analysis, namely extraction, purification and determination. Test extracts have to be cleaned up before instrumental analysis to remove co-extracted materials that often interfere with their determination. Since mycotoxins are such a diverse group of chemical compounds, and are present in a wide variety of food items it is difficult to find a simple procedure, which specifically removes all "interfering" components whilst leaving the mycotoxins in the extract. Common clean-up techniques, which have been used, are:

(i) Liquid-liquid extraction: This is commonly used, often in conjunction with one of the other clean-up procedures, to provide additional clean-up and also to transfer toxins from one solvent system to another whilst at the same time considerably pre-concentrating the toxins. The extraction is carried out in a separating funnel, which contains two immiscible solvents. The solvents are selected so that the mycotoxins are preferentially partitioned into one of the solvents. Proper attention to the choice of solvents is necessary in order to minimize the risk of emulsion formation.



(ii) **Solid-Phase Extraction (SPE):** Recent developments due to environmental hazards and toxicity associated with use of organic solvents such as chloroform, methanol, and acetonitrile in large quantities in conventional clean-up techniques, has seen an increase in the use of disposable columns. Normal SPE columns act by retaining the analyte on the absorbent, eluting the co- extracted non-aflatoxin material, and finally eluting the aflatoxins. Recent developments have seen the use of affinity columns and multifunctional SPE columns such as Multifunctional columns (MFC), and Isolute Multimode columns (IMC). The dense solid phase packing material in these columns not only retains large molecules, but also more importantly contains several types of active lipophilic (non-polar) and charged (polar) sites to adsorb various classes of fats, xanthophylls pigments, chemicals, carbohydrates and proteins. Interfering compounds are removed in less than 30 seconds and at the same time allow compounds of interest to pass through, in a 1-step purification process. (Wilson and Romer, 1991). The MFC column differs from the affinity columns and normal solid phase extraction (SPE) columns that have been used extensively for mycotoxin extract purification. Both the affinity column and the SPE column clean-ups require 3 steps for extract purification: retention of the mycotoxin on packing material of the column, washing away undesirable compounds, and eluting compounds of interest. An MFC column cleanup procedure requires only one-step with no wash or elution solvents. Recovery of total mycotoxins through Multifunctional columns is typically between 85-100% (Wilson and Romer, 1991; Ali *et al.*, 1999; Akiyama *et al.*, 2001).

## 1.7 Objectives of the study

Mycotoxins are such a diverse group of chemical compounds that it is extremely difficult to find a simple procedure, which specifically removes all non- mycotoxin "interfering" compounds whilst leaving the mycotoxins in the extract. Recently there has been a substantial increase in the use of Multifunctional clean-up columns due to the advantages of speed, ease of use and cost. The Multifunctional cleanup column (MFC) has recently been developed for liquid chromatographic determination of aflatoxins in agricultural products (Wilson & Romer, 1991), and in spices (Akiyama *et al.*, 2001). Akiyama *et al.* (1996) have recently reported the analysis of aflatoxins in corn and peanut products using the Isolute Multimode Column (IMC). In a comparative study conducted by Ali *et al.* (1999), the performance of the IMC, and MFC with the official AOAC HPLC method in the analysis of aflatoxins in commercial foods from Malaysia and the Philippines was evaluated. The IMC was found to be the most rapid, and cost-effective and the most efficient. It should be pointed out that Akiyama *et al.* (1996) developed the method for the analysis of peanuts and corn then later applied it to spices.

The objective of this project is to investigate the suitability of the method described by Akiyama *et al.* (1996) for the analysis of aflatoxins in traditional medicine preparations. The feasibility of the Isolute Multimode Column (IMC) for the analysis of traditional medicine preparations will also be investigated. Medicinal plants are the oldest known health-care products and are also important for pharmacological research and drug development. In South-east Asia and Africa, there is a significant use of traditional medicines in the form of preparations packaged in different forms. It would therefore be pertinent at this point to study levels of contamination of some of these products by

aflatoxins, as very limited and scattered reports have been published on these items (Hitoko *et al.*, 1978; Llewellyn *et al.*, 1981).

In this study, a total of 35 different samples have been taken from three Southeast Asian countries namely Malaysia, Indonesia and Thailand (in the form of packaged traditional medicine preparations) and one African country namely Nigeria. They were analysed using a slight modification of the Akiyama method incorporating the IMC.

## **CHAPTER 2**

### **EXPERIMENTAL**

#### **2.1 Materials and Methods**

For this study, a total of 35 traditional medicine samples were analysed, comprising of 12 samples from Malaysia, 10 samples from Indonesia, 8 samples from Thailand, and 5 samples from Nigeria. The samples from Malaysia, Indonesia, and Thailand were in the form of pre-packaged traditional medicine preparations, while those from Nigeria were in the form of un-packaged traditional medicines. Samples were purchased at pharmacy shops. Samples obtained from Nigeria were carried via person and stored immediately in a freezer on arrival, along with other samples. A list of samples studied is shown in Tables 2.1- 2.4.

#### **2.2 Chemicals and Aflatoxin standards**

Methanol and acetonitrile used (Fischer Scientific Co. UK) were HPLC grade, and ultra pure water was from an SG Water Treatment System, SG Wasseraufbereitung GmbH, Germany. Aflatoxin standards and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Isolute Multimode <sup>TM</sup> Multimode (3 ml capacity, 100 mg mass) columns were purchased from International Sorbent Technology, Mid Glamorgan, UK.